

SUPPLEMENTAL MATERIAL

Materials and Methods

Patients and Sample Collection: 38 patient samples were selected from the Oxford Vascular Study (OXVASC) cohort – a population-based study of all acute vascular events in approximately 92,000 residents of Oxfordshire. Briefly, patients were selected who had suffered an ischaemic stroke with an NIHSS score of >5 (i.e. a major ischaemic stroke) and had blood taken within 24 hours of symptom onset. Further selection criteria included inclusion in an OXVASC study investigating serum levels of various biomarkers, including C-reactive protein. All samples were spun and frozen within 2 hours of sampling and were stored at -80°C. Blood samples from healthy controls were also collected in OXVASC (usually from friends or spouses of patients) and samples were processed and stored in the same way. For the current study, controls were matched to ischaemic stroke cases by sex and by age to within 6 months.

Extracellular Vesicle (EV) Isolation for Nanoparticle Tracking Analysis (NTA): 100µl of serum was combined with 900µl of EV-free phosphate buffered saline (PBS) and centrifuged at 34,000rpm for 1 hour to isolate the extracellular vesicle fraction. This was re-suspended in 100µl PBS and further diluted 1:100 in PBS for nanoparticle tracking analysis (NTA).

NanoSight Tracking Analysis (NTA): A NanoSight LM10 (NanoSight Ltd., Amesbury, United Kingdom), was used to count and determine the mean size of the isolated particles. The system uses a finely focused laser beam that is introduced to the sample through a glass prism. The beam refracts at a low angle as it enters the sample, resulting in a thin beam of laser light that illuminates particles through the sample. Particles resident within the beam are visualized using a conventional optical microscope, fitted with a video camera, aligned normally to the beam axis, which collects light scattered from all particles in the field of view. The sample chamber is 500µm deep, but the beam depth is around 20µm at the point of analysis, matching with the depth of focus of the imaging optics. A video of typically 60 seconds duration is taken, with a frame rate of 30 frames per second, and particle movement is analyzed by NTA software (NanoSight Ltd.). The NTA software is optimized to first identify and then track each particle on a frame-by-frame basis, and its brownian movement tracked and measured frame to frame. The velocity of particle movement is used to calculate particle size by applying the two-dimensional Stokes-Einstein equation:

$$\langle x, y \rangle^2 = \frac{K_B T_{ts}}{3\pi\eta d_h}$$

Where $\langle x, y \rangle^2$ is the mean squared displacement, K_B is Boltzmann's constant, T is the temperature of the solvent in Kelvin, t_s is the sampling time (i.e., 1/30 fps = 33 msec), η is the viscosity, and d_h is the hydrodynamic diameter.

Western Blotting: Total protein was extracted from vesicle fractions using RIPA buffer and quantified using a micro BCA kit (Thermo Scientific, UK). 25µg total protein was loaded for electrophoresis and transferred onto a PVDF membrane. Blotting was performed overnight using antibodies against vesicle markers Tsg101 (4A10 Ab83; AbCam, Cambridge, UK; 1:250 in 5% BSA), Alix (3A9 ab117600; AbCam; 1:500 in 5% milk) and CD9 (ExoAb-1; Stratech, Suffolk, UK; 1:1000) in both stroke and control samples. Detection was performed using horse-radish peroxidase conjugated secondary antibodies (1:10,000) and blots were visualized using chemiluminescence and a ChemiDoc (Bio-Rad, UK).

Protein Digest: 90µl of EVs from the NTA were spun at 120,000g for 2 hours to deplete serum proteins from the pellet. The pellet was briefly air dried and frozen on dry ice. Frozen EV pellets were re-suspended in 100µl lysis buffer (ThermoFisher, Loughborough, UK) with 20mM DTT and incubated for 30 minutes at room temperature. Samples were then diluted to 180µl with ultra-pure water and

alkylated with 20µl 200mM iodoacetamide for 30 minutes at room temperature. Proteins were precipitated using methanol/chloroform extraction as described elsewhere.¹ Precipitated proteins were re-suspended in 50µl 6M urea and protein digestion was carried out with 0.6µg trypsin (Promega, Southampton, UK) after dilution to 1M urea with ultra-pure water. Samples were desalted on C18 solid-phase extraction cartridges (SOLA C18, Thermo), dried and re-suspended in 2% acetonitrile 0.1% formic acid for analysis by LC-MS/MS.

Liquid Chromatography – Mass Spectrometry/Mass Spectrometry (LC-MS/MS): LC-MS/MS analysis was performed in technical duplicates using a Dionex Ultimate 3000 UPLC coupled on-line to a Q Exactive HF mass spectrometer (Thermo Scientific). Samples were separated on an EASY-Spray PepMap C18 column (500mm x 75µm, 2µm particle size, Thermo Scientific) over a 60 minute gradient of 2-35% acetonitrile in 5% DMSO 0.1% formic acid at 250nl/min. MS1 scans were acquired at a resolution of 60,000 at 200 m/z and the top 15 most abundant precursor ions were selected for HCD fragmentation.

Protein Quantification: Protein quantitation was performed using Progenesis Q1 for Proteomics (Non-linear Dynamics, version 2.0). MS/MS data was searched using Mascot (Matrix Science, version 2.5.1) against the human Swissprot database (retrieval date 15.10.14) allowing 1 missed cleavage. Mass tolerances were 10ppm for precursor and 0.05 Da for fragment masses. Carbamidomethylation of cysteine was set as a fixed modification. Oxidation of methionine, deamidation of asparagine and glutamine were set as variable modifications. Identified peptides scoring below 20 following application of a 1% false discovery rate were discarded and the Mascot search results were imported back into Progenesis for label-free protein quantitation.

Cell Culture: Human monocyte cell line (THP-1) were maintained under normal cell culture conditions (37°C; 95% CO₂; 5% air) in RPMI supplemented with 10% FCS, L-glutamine, and antibiotic/antimycotic and 2-mercaptoethanol. For stimulation paradigms cells were plated in 6-well plates at 1 x 10⁶ cells per well and differentiated into macrophages with phorbol 12-myristate 12-acetate for 24 hours. EVs from clinical stroke samples and age-matched controls were added to cells and allowed to incubate for 4 hours. Analysis was performed on whole RNA for expression levels of inflammatory cytokines and chemokines.

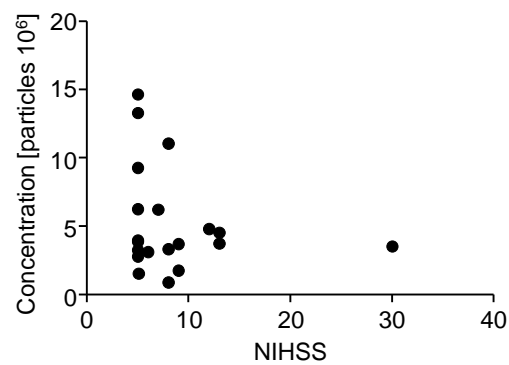
RNA Extraction and qPCR: RNA was extracted from whole cells according to manufacturers' instructions (QIAGEN, UK) and converted to cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems, UK). qPCR was performed using the SYBR green based technology (PrimerDesign Ltd., UK) and analysed using the Pfaffl method.² Expression levels were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase and normalised to expression levels in control cells.

Statistics: The normalised intensity values of the protein concentrations were converted into z scores for subsequent analysis. The heat map and hierarchical cluster analysis was generated using the heatmap.2 (gplots, version 3.0.1) function in Rstudio (version 0.99.893). Clustering was performed in both the x and y direction to investigate the clustering of the samples and the individual proteins. All other data were analysed using Prism 6.0 and presented as mean ±SEM with data being considered significant at p<0.05.

References

1. Wessel D, Flugge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem.* 1984;138:141-143
2. Pfaffl MW. A new mathematical model for relative quantification in real-time rt-pcr. *Nucleic Acids Res.* 2001;29:e45

Supplemental Figure I



Supplemental Figure I: Extracellular vesicle number plotted against NIHSS score. Data points represent individual patients. n=17.